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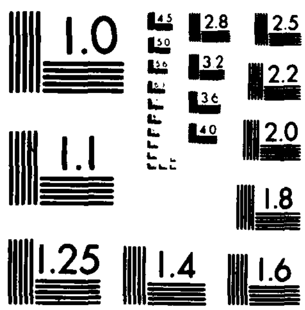
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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGB_x EFFECT

II. REACTION MEDIUM COMPONENT REQUIREMENTS FOR PGB_x EFFECT

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The in vitro system for the assay of the PGB_X effect was investigated by exposing RLM to the individual components of the normal system, as well as to all possible binary mixtures of these constituents. The degree of degradation, as measured by the decrease in absorbance at λ525nm, was compared with the ability of RLM to carry out oxidative phosphorylation. The results indicate there is no correlation between the PGB_X effect and the effect of PGB_X on the swelling of RLM.

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INTRODUCTION

The possible use of PGB_x (1, 2, 3) as a therapeutic agent in the treatment of human ischemic diseases was suggested by the favorable results from only a few animal experiments (4, 5, 6, 7, 8, 9, 10). Obviously before any human trials may be undertaken much more animal testing must be completed, and in addition, certain basic information concerning the mechanism of action of this drug must be known. At this stage of our knowledge of PGB_x, the possibility of the successful elucidation of the *in vivo* mechanism of action appears remote. However, since the elucidation of the *in vitro* mechanism of PGB_x might reveal an insight as to the *in vivo* mechanism, studies have been underway to define the *in vitro* PGB_x effect.

In previous reports (11) it was shown that the PGB_x effect could only be demonstrated with isolated RLM exposed to the hypotonic conditions described by Polis *et al* (2). Since this "degradation" of RLM is a prerequisite for the demonstration of the PGB_x effect, the composition requirements of the degradation medium was studied in detail. This report describes the results of varying the composition of the PGB_x assay system on the demonstration of the PGB_x effect.

METHODS AND MATERIALS

PGB_x Type II prepared and assayed as described previously (2) was used in this study. The composition of the degradation stage was varied as indicated in the legends of the figures. In all cases the volume was 2.02 ml and the concentration of each component of the mixture was the same as that in the normal assay. At the end of the degradation stage, the constituents of the normal assay not added in the specific degradation test, were then added with the nucleotide mixture. The degradation time was determined by exposing the RLM to the specific degradation mixture for various times, usually 5-50 minutes, then adding the nucleotide mixture (stage 2) and allowing the reaction to proceed for 20 minutes. At this time the reaction was stopped by adding $HClO_4$ and Pi concentration of the protein free filtrate was analysed as described previously (2, 3). In all cases 4.0mg RLM were used.

Note: Abbreviations used in this report are: RLM, rat liver mitochondria; Pi, inorganic phosphate; BSA, bovine serum albumin

The swelling and contraction of RLM exposed to the stages of the PGB_x assay were measured by absorbancy changes at λ525nm. The absorbancy was measured with a spectrophotometer (Bausch and Lomb Spectronic 100, Rochester, NY) and the output recorded on a 100 mv strip chart recorder (Laboratory Data Control, Riviera Beach, FL).

EXPERIMENTAL

In order to demonstrate the *in vitro* PGB_x effect on RLM oxidative phosphorylation, the RLM must first be deactivated by exposure to state 4 respiratory conditions (12). If the RLM were first exposed to state 3 respiratory conditions, no PGB_x effect was observed because the degree of phosphorylation was too high. It was of interest then, to study the effect of varying the composition of the degradation mixture in an attempt to ascertain those components involved directly in the PGB_x effect, and in this way possibly ascertain the mechanism of the *in vitro* PGB_x effect. As a reference point the degrading medium described by Polis *et al* (2) and listed in Table I was first used in all the studies.

TABLE I

The Composition of the Medium for the Demonstration of the PGB_x Effect on Mitochondrial Oxidative Phosphorylation

Order of Addition	Mitochondrial Degrading Medium	Reaction Mixture
Water	1.55 ml	1.55 ml
Phosphate Buffer pH 7.35	4.98 mM	4.55 mM
α-Ketoglutarate pH 7.35	14.93 mM	13.64 mM
$MgSO_4$	4.98 mM	4.55 mM
Aged Mitochondria	1.99 mg/ml	1.82 mg/ml
Sucrose*	5.97 mM	5.45 mM
EDTA*	0.010 mM	0.009 mM
AMP	-----	2.27 mM
ADP	-----	2.27 mM
KCl	-----	45.45 mM
Bovine Serum Albumin	-----	0.68 mg/ml

Total Volume: 2.20 ml   Degradation Time: 5-20 minutes
Temperature: 28°   Reaction Time: 20 minutes

*Added with mitochondria

To define the optimal conditions for the PGB_x effect, RLM was exposed to solutions of the individual components of the normal degradation mixture as well as all possible mixtures of the three constituents. In addition, RLM was exposed to water only. The degree of RLM degradation was evaluated by measuring the residual ability of RLM to phosphorylate ADP under respiratory state 3 conditions. In preliminary experiments it was found that the residual RLM phosphorylation ability varied considerably depending upon the composition of the degradation medium. In order to compare the results of the various degradation media, a constant residual phosphorylation ability of 10% was chosen arbitrarily. The time to yield this level of RLM degradation was determined by measuring the residual phosphorylating ability as a function of time of exposure. When this data was plotted, the time to 10% phosphorylation could be interpolated. Figure 1 shows the residual phosphorylation as a function of degradation time for the normal system, water only, $MgSO_4$ only, phosphate buffer only and α-ketoglutarate only. Figure 1b shows the curves for normal system, α-ketoglutarate plus phosphate buffer, $MgSO_4$ plus phosphate buffer, and $MgSO_4$ plus α-ketoglutarate. The interpolated times to yield 10% phosphorylation by RLM exposed to the various media studied are listed in Table II.

TABLE II

Time Required to Deactivate RLM 90% When Exposed to Hypotonic Media

Medium Composition	Conc. (mM)	Time (minutes)
Normal System		
$MgSO_4$	4.98	10
Phosphate	4.98	
α-ketoglutarate	14.93	
Single Solutions		
Water	-----	15
$MgSO_4$	4.98	20
Phosphate Buffer	4.98	30
α-ketoglutarate	14.93	60
Binary Solutions		
α-ketoglutarate	14.93	40
Phosphate	4.98	
Phosphate	4.98	40
$MgSO_4$	4.98	
α-ketoglutarate	14.93	60
$MgSO_4$	4.98	

It is interesting to note that the degradation of RLM was the greatest when exposed to Polis's normal system, which was even greater than when RLM was degraded in the presence of H_2O only. The medium that yielded the least degraded RLM was with α-ketoglutarate. When Pi was the degradation medium the degree of degradation was also at a low level. When binary mixtures of the three constituents were used, the RLM appeared to be more resistant. Again in the presence of α-ketoglutarate, even after 40 minutes, the RLM never were degraded to the 10% residual phosphorylation level. Using the degradation times listed in Table II for the specific medium, the effect of varying the PGB_x concentration from 2-36μg/ml was measured and plotted. These results are shown in Figures 2A and 2B. A PGB_x effect was observed in all of the media tested, although the intensity and shape varied. In the case of the degradation media composed of individual constituents only, when phosphate buffer was used, the phosphorylation ability of the degraded RLM approached the level obtained with the normal system. When H_2O only, α-ketoglutarate only, and $MgSO_4$ only was used as the degrading medium, a less intense PGB_x effect was observed. In the case of the binary degradation media, when Pi was included in the mixture the PGB_x effect was approximately similar to that obtained with the normal system.

In an effort to correlate the effects of RLM degradation with the integrity of the RLM membrane, the swelling and contraction of RLM was measured during exposure to the degradation stage and to the reaction stage of the PGB_x effect[2,3]. The swelling and contraction were measured by following the absorbancy changes at λ525nm. In Figure 3 the swelling and contraction of RLM exposed to the degradation media of H_2O only, 0.3M sucrose only, the normal control system and the normal PGB_x system (4.55μg PGB_x/ml) are shown. In the normal control system the RLM swell rapidly in the first three minutes and approach a minimum absorbance at the end of the degradation period. With the addition of the nucleotide mixture there is an immediate drop in absorbancy, probably caused by a dilution effect followed by a further slow drop in absorbance. In the presence of PGB_x the absorbancy change in stage 1 is only changed slightly in that the final absorbance is higher than the control. However, upon the addition of the nucleotide mixture there is an immediate increase in absorbancy, indicating a contraction of RLM. This contraction is probably associated with the formation of ATP generated by the PGB_x treated system. In contrast, when 0.3M sucrose was the degradation medium, swelling did not occur either during the degradation stage or during the reaction stage. The drop in absorbancy after the addition of the nucleotides plus the remaining constituent of the assay system is the result of dilution. When water was the degrading medium the large drop in absorbancy

was too rapid to be recorded and only the final change was measured. Upon the addition of the remaining constituents, there was an immediate contraction resulting from the formation of ATP followed by a slow decrease in absorbance.

Figure 4 shows the swelling and contraction of RLM exposed to α-ketoglutarate alone, phosphate buffer alone and $MgSO_4$ alone. When the RLM was exposed to α-ketoglutarate alone there were only minor changes in absorbance during stages 1 and 2. In the presence of inorganic phosphate there was a gradual, continuous swelling during stage 1 with an immediate contraction during stage 2 which was maintained during the course of stage 2. In the presence of $MgSO_4$ alone, during stage 1 the immediate swelling was too fast to be recorded. The swelling then leveled off during the remainder of stage 1. During stage 2 there was an immediate contraction, followed by a slow swelling of the RLM. The swelling and contraction of the RLM exposed to the binary mixtures are shown in Figure 5. The swelling and contraction profiles of RLM in the presence of $MgSO_4$ and Pi, and in the presence of α-ketoglutarate and Pi were similar to that observed with $MgSO_4$ alone (Figure 4). The swelling and contraction profile of RLM in the presence of α-ketoglutarate plus Pi was characterized by a slow swelling during stage 1 that leveled off after about three minutes and during stage 2, only a slight contraction was observed which remained approximately constant.

In addition to the degradation studies of RLM in the presence of the normal PGB_x stage 1 constituents, similar studies were also carried out using the normal stage 2 constituents. Figure 6 shows that in the presence of KCl only, RLM show an immediate contraction followed by no change in absorbance during stage 1; however upon the addition of stage 2 reactants there was an immediate and slow contraction that leveled off after several minutes. In contrast when RLM were exposed to KCl plus PGB_x, the stage 1 absorbance profile was similar; however in stage 2 the swelling was followed by a contraction. When the AMP and ADP mixture was the medium in stage 1, there was a slow swelling of RLM followed by an immediate contraction upon the addition of the stage 2 components. When BSA only was the stage 1 medium the swelling and contraction profile was similar to that of H_2O only, except that the swelling and resultant contraction were at a lower level.

In an attempt to relate the PGB_x effect with possible changes in RLM morphology, the degree of swelling of RLM and the phosphorylating ability after exposure to the various degradation media were summarized in Table III.

TABLE III

The Effect of Various Degradation Media on the Swelling and Phosphorylating Ability of RLM

Composition of Degradation Medium[1]	Degree of Swelling (Δ $^{A}525nm/ml$)[2]	Pi Esterified (mM)
Normal System Control	-0.406	0.18
Normal System + PGB_x (4.95μg/ml)	-0.356	2.73
H_2O only	-0.441	0.69
$MgSO_4$	-0.262	1.38
Pi only	-0.366	2.63
α-ketoglutarate only	-0.144	2.11
$MgSO_4$ plus Pi	-0.262	2.76
KCl only	-0.220	2.13
KCl + $MgSO_4$	-0.025	2.53
AMP + ADP	-0.366	1.32
BSA only	-0.599	1.81

1 Total volume of degradation mixture was 2.02 ml, and contained 4.0mg RLM; the concentration of each constituent was that used in the normal system.

2 Δ $^{A}525nm$ was calculated by subtracting the $^{A}525nm$ of each test after 10 minutes and subtracting this value from that found for $^{A}525nm$ for 4.0mg RLM in 2.02 ml 0.3M sucrose. Negative values indicate swelling.

From these data there appeared to be no relationship between the morphological integrity of the RLM and its ability to phosphorylate. Those conditions that resulted in the least ability of the RLM to phosphorylate, namely H_2O only and the normal control system, showed a high degree of swelling, although not as high as that shown with BSA only. In this latter system the ability of the RLM to carry out phosphorylation was reduced only about 50%. Those media that resulted in the least swelling of RLM, e.g. KCl only and KCl plus $MgSO_4$, only caused a 30% decrease in phosphorylating ability.

DISCUSSION

The results of this study show that the hypotonic medium used by Polis et al[2,3] to degrade RLM is the optimum medium to demonstrate the PGB_x effect. The omission of any one constituent, or the use of only one constituent, results in the RLM not being sufficiently degraded to show the PGB_x effect. However by modifying the experimental conditions so that the RLM are degraded sufficiently, i.e. 10% residual phosphorylating ability, then in all the media studied a PGB_x effect was observed.

The results also point out that the ability of RLM to phosphorylate is not necessarily dependent upon the degree of morphological swelling. The addition of PGB_x to the normal system only induces a minor change in swelling yet a tremendous improvement in phosphorylating ability. When RLM were exposed to media that induced only a minimal swelling, the concomitant phosphorylating ability was not maximal. On the other end of the scale, the medium that induced the greatest swelling, BSA only, still yielded RLM that gave a relatively high phosphorylation activity.

In previous studies it was observed that RLM exposed to the normal hypotonic medium for the required time, when then treated with PGB_x, showed no improvement in swelling nor in phosphorylating ability. From this it was concluded that PGB_x reacts with RLM to protect them from hypotonic swelling. From the results of this study the above explanation appears too simplistic. Although PGB_x must bind to the RLM, it does not inhibit swelling and therefore must perform some other function, which unfortunately is unknown at this time.

Figure (1a): The phosphorylating ability of RLM exposed to hypotonic media as a function of time. RLM (4.0mg) were added to the media at a final volume of 2.02 ml. At the end of each degradation time the nucleotide mixture plus the components of the normal system omitted in each test were then added and the reaction allowed to proceed for 20 minutes. The concentration of the components alone and in the binary mixtures was that listed in Table I. At the end of this time the solution was deproteinized by the addition of $HClO_4$ and the Pi analysed as described previously (2,3). Legend for curves: ., normal system; +, H_2O only; , $MgSO_4$ only; 0, phosphate buffer only; and Δ, α--ketoglutarate only.

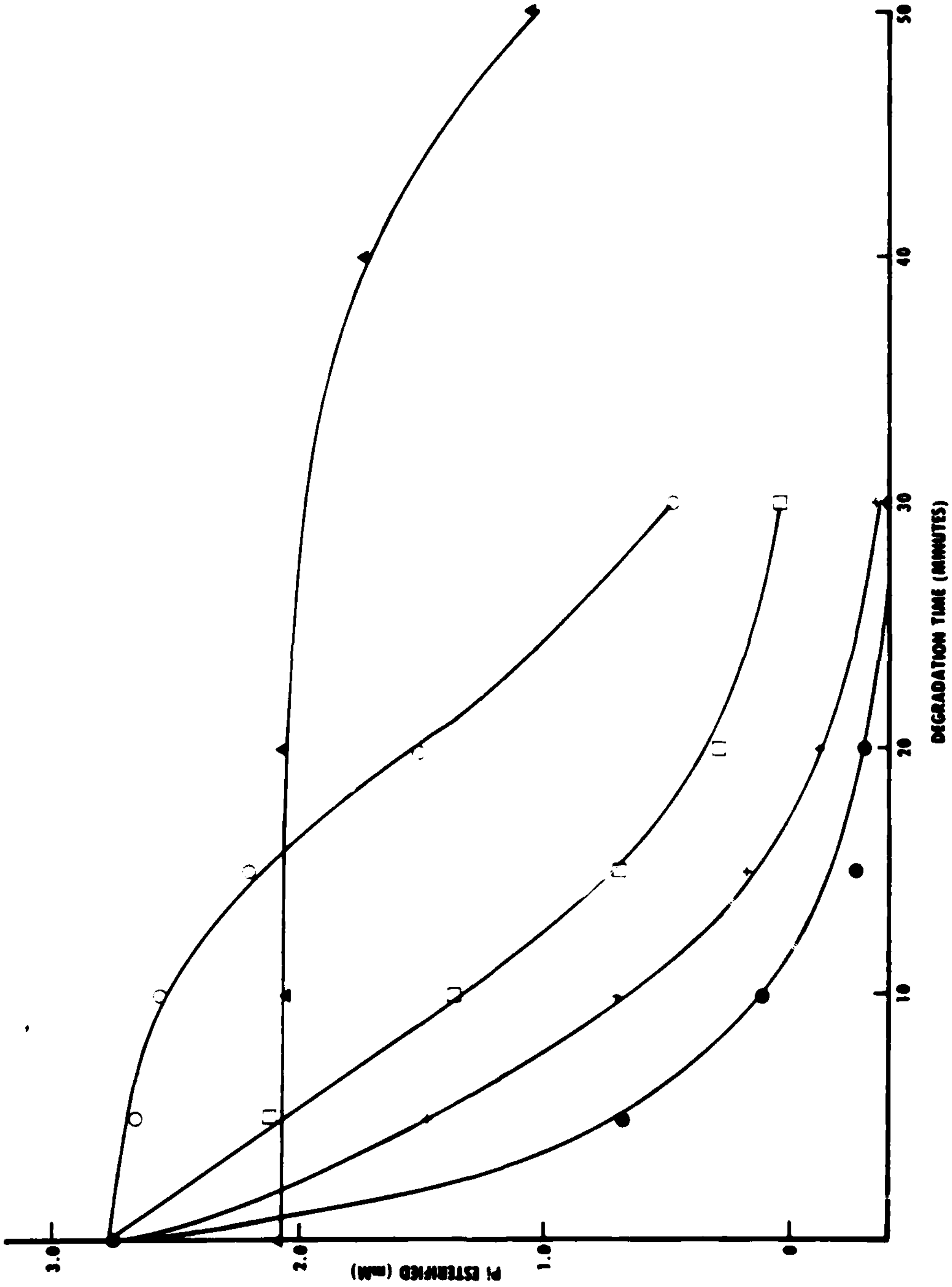

Figure 1a - The Phosphorylating Ability of RLM Exposed to Hypotonic Media as a Function of Time

<u>Figure (1b)</u>: Legend for curves: ., normal system; Δ, α-ketoglutarate and phosphate buffer; 0, phosphate buffer and $MgSO_4$; and , α-ketoglutarate and $MgSO_4$.

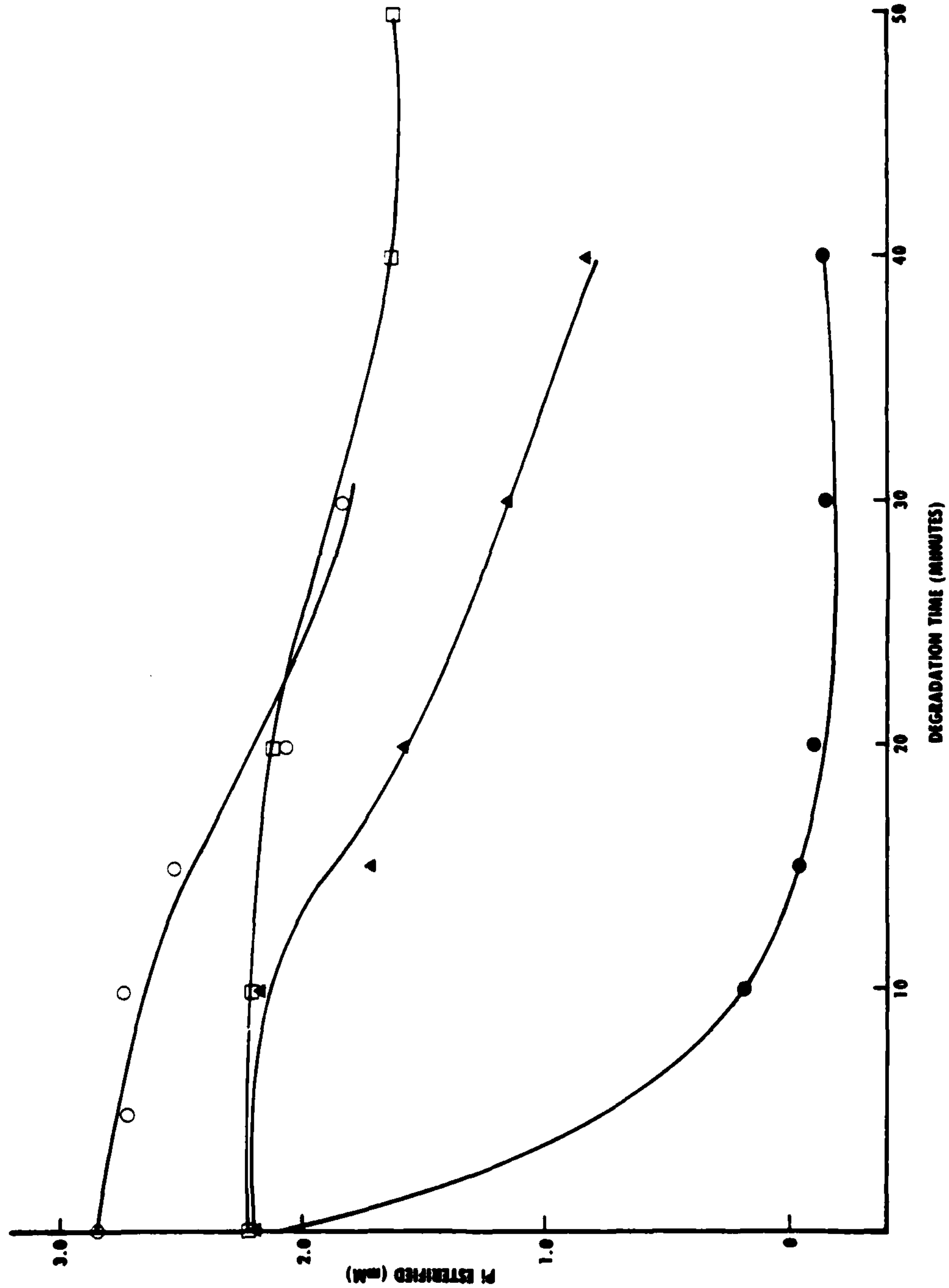

Figure 1b - The Phosphorylating Ability of RLM Exposed to Hypotonic Media as a Function of Time

<u>Figure (2a)</u>: The effect of added PGB_x on the phosphorylating ability of RLM after exposure to various hypotonic media. RLM (4.0mg) were added to the hypotonic containing various amounts of PGB_x. The final volume of the degradation media was 2.02 ml. The degradation times used were calculated to yield less than 10% residual phosphorylation by the degraded RLM (Table II). After the addition of stage 2 reactants the Pi remaining was analysed as described previously (2,3). Legend for curves: ., normal system; +, H_2O only; , $MgSO_4$ only; 0, phosphate buffer only; and Δ, α-ketoglutarate only.

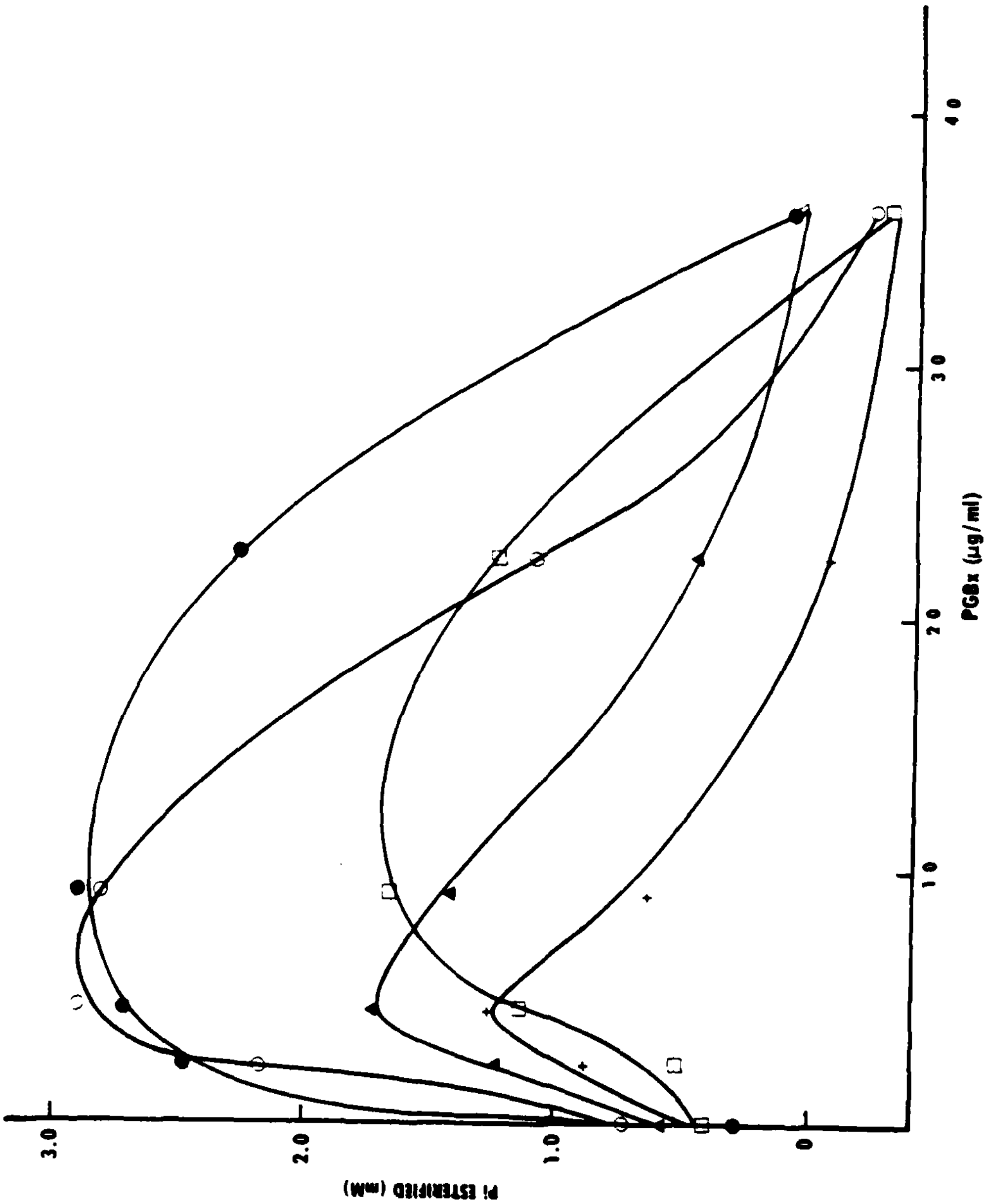

Figure 2a - The Effect of Added PGB_x on the Phosphorylating Ability of RLM After Exposure to Various Hypotonic Media

<u>Figure (2b)</u>: Legend for curves: ., normal system; 0, $MgSO_4$ and phosphate buffer; , α-ketoglutarate and $MgSO_4$; and Δ, α-ketoglutarate and phosphate buffer

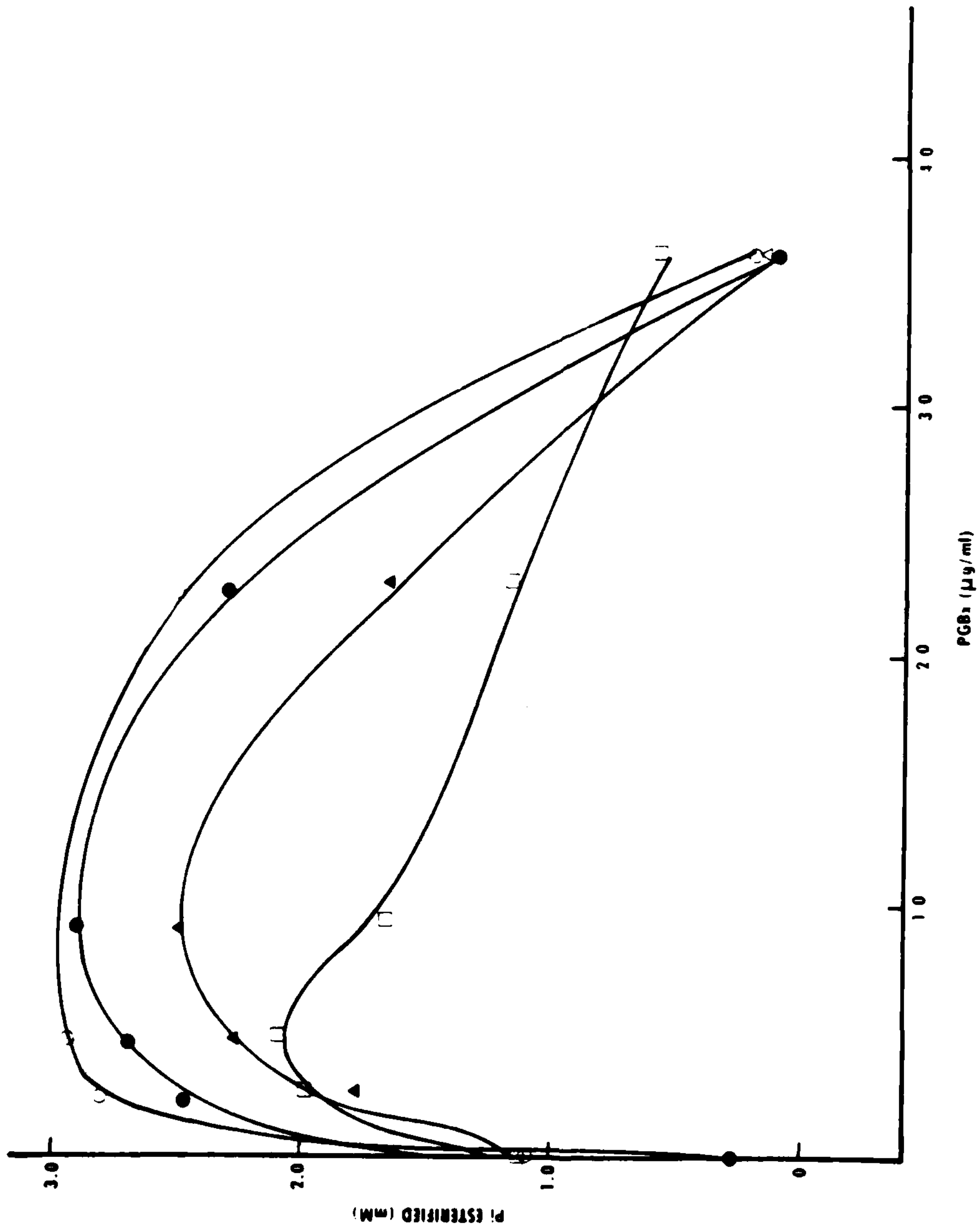

Figure 2b - The Effect of Added PGB$_X$ on the Phosphorylating Ability of RLM After Exposure to Various Hypotonic Media

<u>Figure (3)</u>: The changes in absorbance at $\lambda 525nm$ for RLM exposed to various hypotonic media during stage 1 and stage 2 of the PGB_x assay system. (See Table I for the concentration of the reagents used). RLM (4.0mg) were added to hypotonic media (total volume, 2.02 ml) was the $A_{\lambda 525nm}$ recorded as a function of time. After 13 minutes stage 2 reactants were added as indicated by the break in the curves, and the $A_{\lambda 525nm}$ recorded for an additional 13 minutes. Legend for curves: _____, normal system; ---, normal system plus 10μg PGB_x; 0, H_2O only; and ×, 0.3M sucrose containing 5×10^{-4}M EDTA.

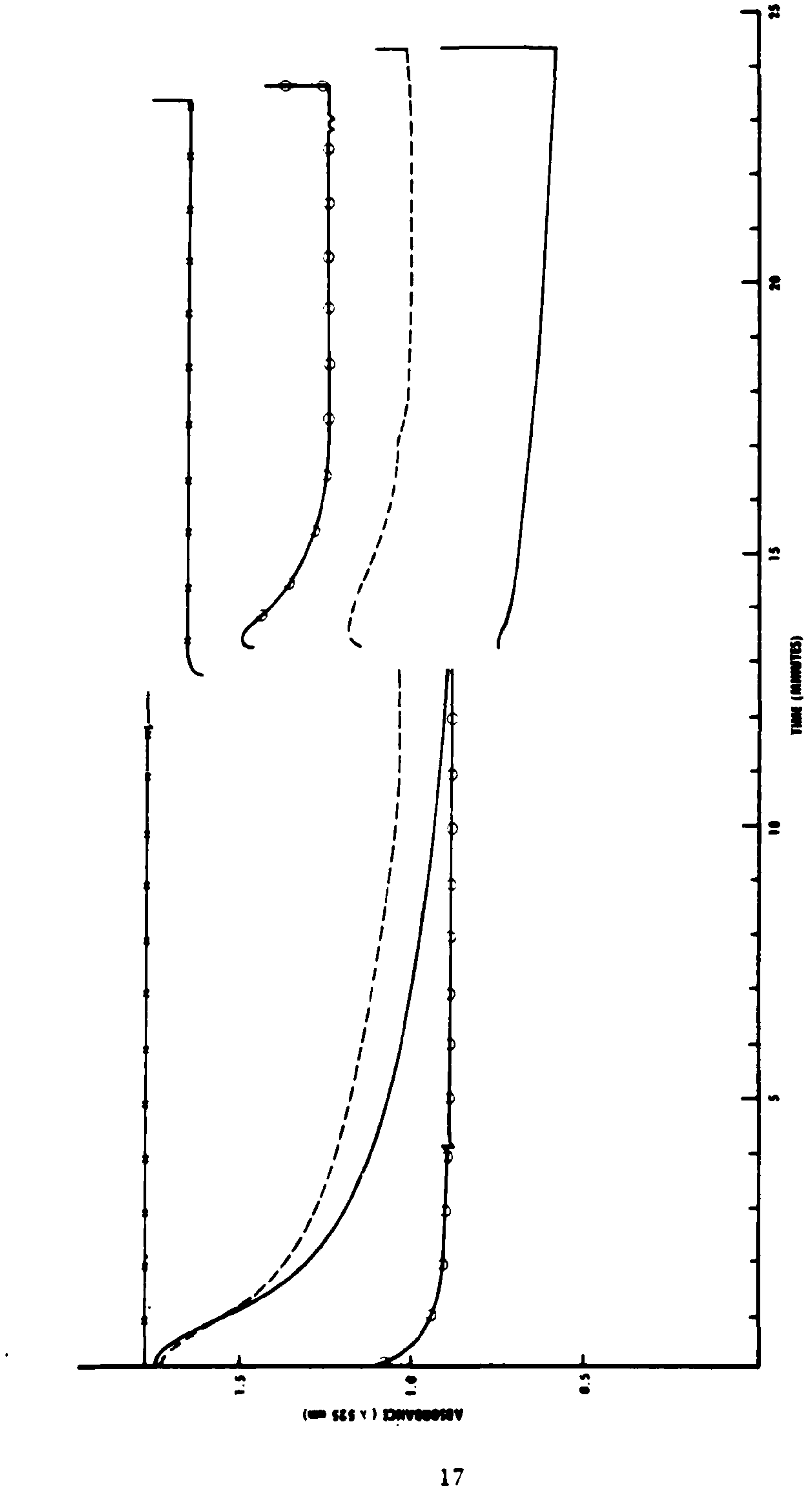

Figure 3 - The Changes in Absorbance of λ525nm for RLM Exposed to Various Hypotonic Media During Stage 1 and Stage 2 of the PGB_x Assay System

Figure (4): See Figure 3 for details. Legend for curves: ---, α-ketoglutarate only; _____, phosphate buffer only; ×, $MgSO_4$ only.

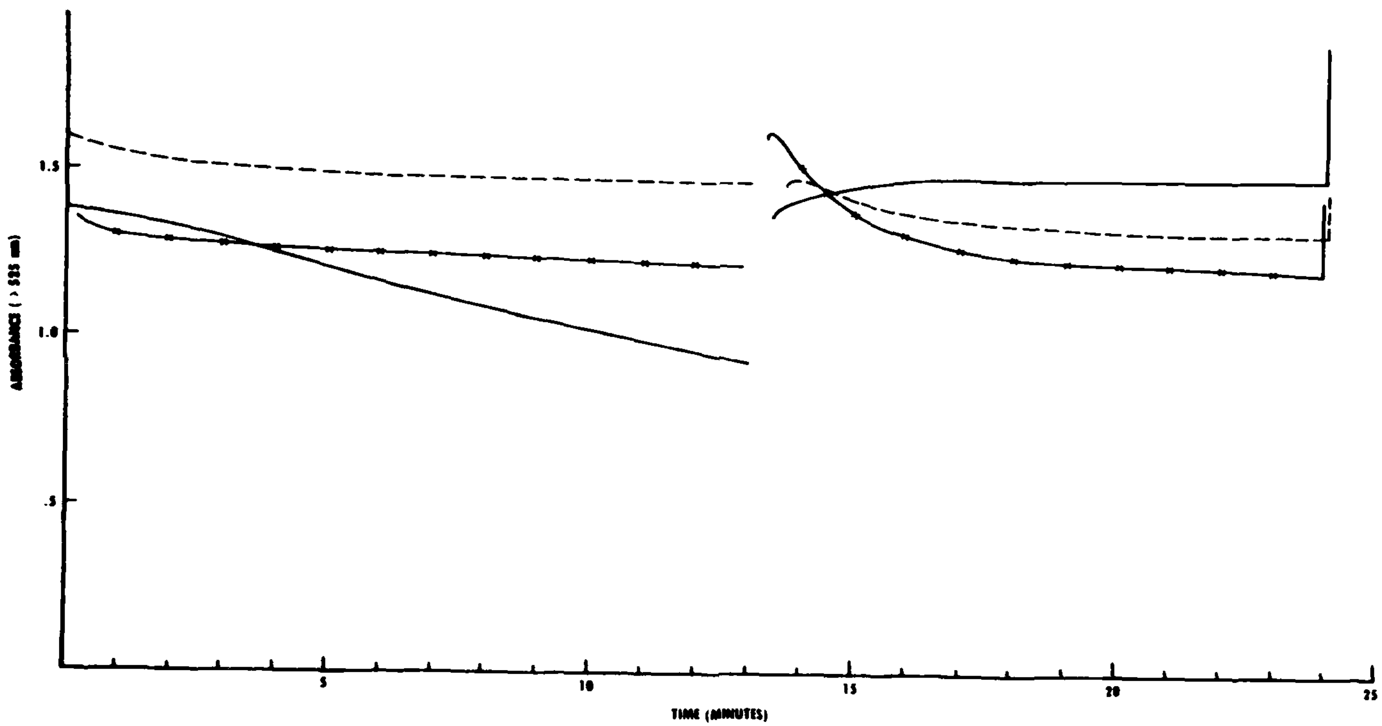

Figure 4 - The Changes in Absorbance of λ525nm for RLM Exposed to Various Hypotonic Media During Stage 1 and Stage 2 of the PGB_x Assay System

Figure (5): See Figure 3 for details. Legend for curves: _____, α-ketoglutarate and phosphate buffer; ---, $MgSO_4$ and α-ketoglutarate; and ×, $MgSO_4$ and phosphate buffer.

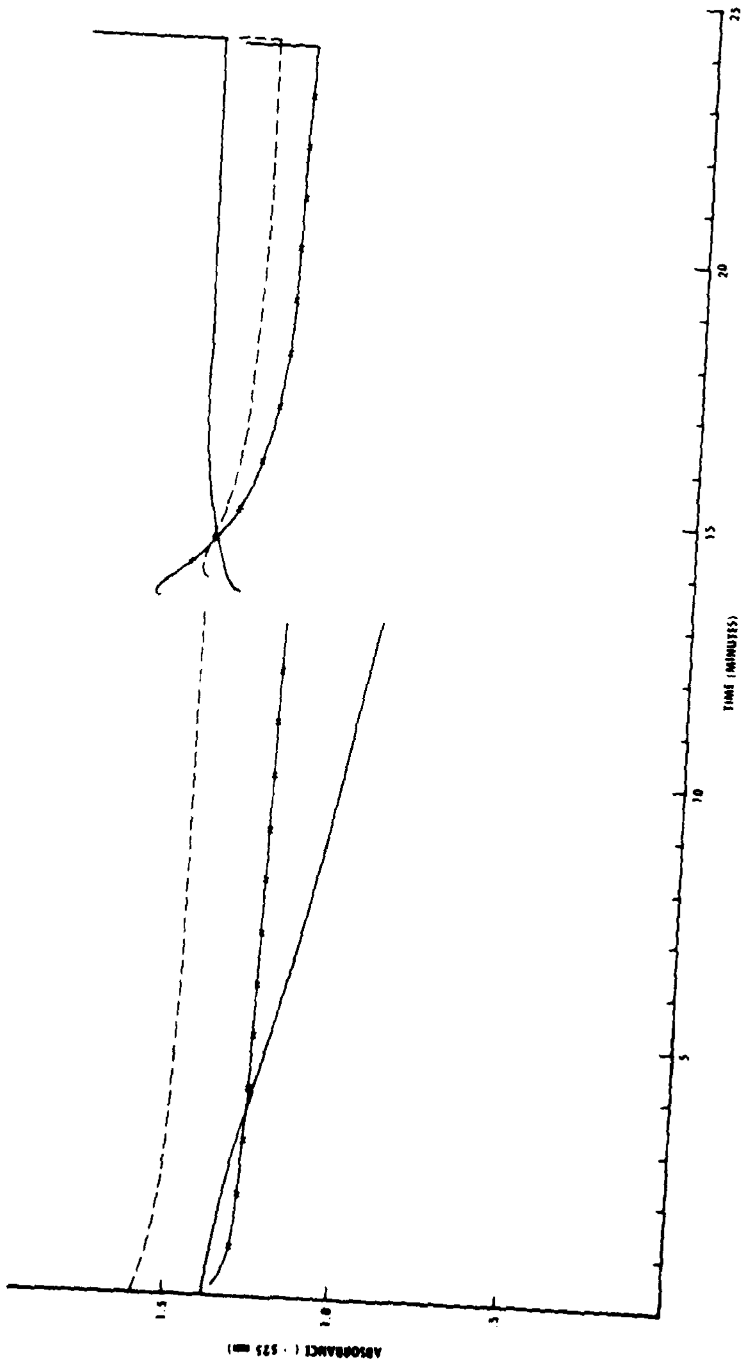

Figure 5 - The Changes in Absorbance of λ525nm for RLM Exposed to Various Hypotonic Media During Stage 1 and Stage 2 of the PGB_x Assay System

<u>Figure (6)</u>: See Figure 3 for details. Legend for curves: ——, KCl; ---, KCl and PGB_x (10μg); ×, nucleotide; and 0, BSA.

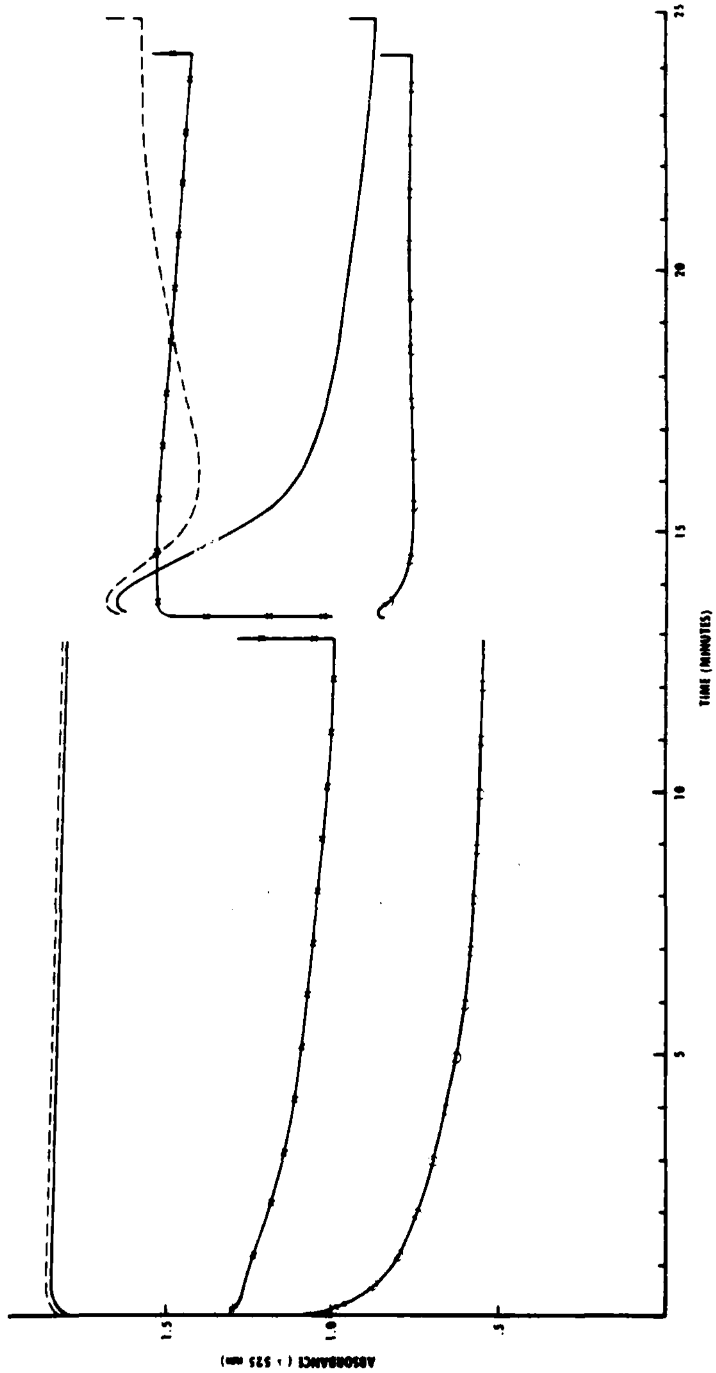

Figure 6 - The Changes in Absorbance of λ525nm for RLM Exposed to Various Hypotonic Media During Stage 1 and Stage 2 of the PGB_x Assay System

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Naval Biosciences Laboratory, Building 844, Naval Supply Center, Oakland, CA 94625	1
Army Research Office, P.O. Box 12211, Research Triangle Park, NC 27709	1
Air Force Office of Scientific Research, Directorate of Life Sciences, Bolling Air Force Base, Washington, DC 20332	1
Army Medical Research & Development Command, Forrestal Building, Washington, DC 20314	1
Department of the Army, U.S. Army Science and Technology Center, Far East, APO, San Francisco, CA 96328	1

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